(12) EUROPEAN PATENT APPLICATION

(54) Processes for the detection of non-A, non-B hepatitis and a kit for use in the same.

(57) There is disclosed a process for assaying a tissue specimen for non-A, non-B, hepatitis antigen. The process comprises:

(a) providing a tissue specimen to be assayed;

(b) contacting the tissue specimen with a non-A, non-B hepatitis antibody;

(c) contacting the resultant tissue specimen with a biotin-avidin system; and

(d) determining the presence of non-A, non-B hepatitis antigen present in the tissue sample.

Also described in an analogous process for assaying for non-A, non-B hepatitis antibody in a biological sample.

A diagnostic kit and a fixed, embedded tissue sample are also disclosed.

EP 0 154 392 A2

PROCESSES FOR THE DETECTION OF NON-A, NON-B HEPATITIS AND A KIT FOR USE IN THE SAME

The present invention relates to a method for the detection of non-A, non-B hepatitis agents and in

5   particular to a method of detecting non-A, non-B hepatitis agents using a tissue screen technique. The tissue screen technique uses an enhanced identification method which renders the inventive procedure a useful diagnostic testing system.

10   Non-A, non-B hepatitis (NANB) has been recognized as a virus-induced disease, distinct from other forms of viral-associated liver diseases, including hepatitis A (HAV) and B (HBV), and the hepatitides induced by cytomegalovirus (CMV) or Epstein-Barr virus (EBV).

15   Several attempts have been made to identify specific NANB antigen-antibody reactions using a variety of methods. To date, neither specific non-A, non-B antigen nor non-A, non-B antibody have been identified. Similarly a number of NANB associated ultra-structural

20   changes and virus-like particles have been described. However, the NANB virus has not yet been definitively identified.

U.S. Patent 4,356,164 to Tabor describes a method of assaying for the amount of non-A, non-B hepatitis

25   antigen present in a sample comprising a first step of reacting antigen in the sample with the antibody, a second step of separating reactive phase from non-reactive phase by precipitating antibody from serum and purifying said antibody, and a third step of

30   measuring the extent of the reaction as a measurement of the amount of non-A, non-B hepatitis antigen present in the sample. Tabor purports to disclose the identification of non-A, non-B hepatitis antigen using counterelectrophoresis (CEP) which Tabor also describes

35   as immunoelectrosmophoresis (IEOP) or immunoelectro-odiffusion (IED) or countercurrentelectrophoresis.

The techniques involved testing sera using 1%

agarose in barbital buffer, pH 8.6, poured onto 3.5 x 12.5 cm glass plates. Melted agarose was then poured onto a lantern slide. When it had cooled, two rows of holes were punched in the agarose. Antibody was placed

5   in one row of holes and samples to be tested were added to the other row. When testing for antibody, antigen was added to one row and samples in the other row. The lantern slide was placed in a CEP chamber. Paper wicks were used to connect each side of the slide to each of

10 two pools of barbital buffer, pH 8.6. An electric current was passed across the plate, 35 milliamps per plate, for one hour. Immunoprecipitin lines were read after 1, 24, and 48 hours of storage in a moist chamber at room temperature. When the test sample was

15 positive, a precipitin line was seen between the rows, using the naked eye with the aid of an electric lamp. The diagnostic technique described is extremely time consuming, subject to erroneous interpretation and does not necessarily represent an antigen-antibody reaction.

20   In contrast, International Patent Application No. PCT/US82100166 to Maupas discloses the isolation from serum of two non-A, non-B hepatitis virus particles.

     Maupas describes the first particle as resembling a togavirus. It is a spherical, coated virus having an

25 outer diameter of 50-60 nm and a core having a diameter of about 40 nm. Heating an aqueous suspension of this virus at 25° C for extensive periods or exposing the virus to ether causes it to lose its infectivity for tissue culture. It was recovered from the plasma or

30 urine of patients suffering from NANB hepatitis.

     The second particle Maupas describes resembles an enterovirus. This enterovirus-like particle is spherical, having a diameter of about 27 nm; exhibits a buoyant density of 1.36 in isopyenic density-gradient

centrifugation with cesium chloride; loses its tissue
culture infectivity upon heating in aqueous suspension
for 3 hours at 90° C, but not after 10 hours at 56° C;
it is insensitive to ether; and its genetic material is
5   positive to a histochemical stain for RNA.

Both particles have been cultured in vitro in
tissue culture.

Even with the prior art developmental activities,
a need still exists for a sensitive, specific method
10 for screening and identification of NANB hepatitis.
NANB infection has been implicated in 60-90% of post-
transfusion hepatitis cases. The risk of contracting
NANB hepatitis after blood transfusion has been
estimated to be close to 10% in the U.S. Therefore,
15 it is important to screen the blood of potential donors
to detect NANB hepatitis. Further, based upon studies
in chimpanzees, the chronic sequelae associated with
NANB-hepatitis now appear to be more serious than
chronic disease developments arising from HBV infection.

20 There has been discovered an unexpected process
which uses a tissue screen method for determining the
presence of non-A, non-B hepatitis agents and particu-
larly antibodies and antigens, which is extremely
sensitive.

25 More particularly the invention involves a process
for assaying a tissue specimen for non-A, non-B hepatitis
antigen by obtaining a tissue specimen which may contain
non-A, non-B hepatitis antigen, contacting the tissue
specimen with a non-A, non-B antibody, contacting the
30 resultant tissue specimen with a biotin - avidin system,
and determining the presence of non-A, non-B hepatitis
antigen present in the tissue sample.

In another embodiment the invention involves a
process for assaying for non-A, non-B hepatitis anti-
35 body present in a biological sample by contacting a
tissue specimen containing non-A, non-B hepatitis

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antigen with the biological specimen to permit reaction of the specific specimen antibodies with the non-A, non-B hepatitis antigen, incubating the reactants for up to 24 hours at temperatures up to 45° C, separating

5 the reactive tissue specimen from the non-reactive phase, contacting the resultant tissue specimen with a biotin-avidin enhancement system, and determining the presence of non-A, non-B hepatitis antibody in the sample.

10 An additional embodiment involves a process for assaying for non-A, non-B hepatitis antigen present in a biological sample, by reacting antigen present in the sample with non-A, non-B hepatitis antibody, separating reactive phase from non-reactive phase by precipitating

15 immune-complexed antibody antigen and recover the fluid-supernatant, contacting a tissue specimen containing non-A, non-B antigen with the fluid supernatant to permit reaction of any non precipitated non-A, non-B hepatitis antibodies with the non-A, non-B hepatitis

20 antigen, incubating the reactants for up to 24 hours at temperatures up to 45° C, contacting the resultant tissue specimen with a biotin - avidin system, and determining the presence of non-A, non-B hepatitis antigen in the sample.

25 The present test method is extremely useful in screening patients serum prior to use of the blood for transfusion purposes; diagnosis of non-A, non-B hepatitis infections; detection of non-A, non-B hepatitis antigen and antibody in biological samples;

30 and liver tissue screening for the presence of antigen. Such procedures have been heretofore not possible with previously reported techniques for detection of low levels of non-A, non-B hepatitis antigen and antibody.

As used herein, the term "biotin-avidin" system

35 refers to a particular enhancement marker for the identification of the presence or absence of a

particular analyte, such as antigen or antibody. Such
systems are generally well known and do not constitute
an inventive aspect of this invention other than by their
critical use to detect NANB agents. Such techniques

5 have been known for detecting herpes simplex virus, see
Nerurkar et al (1983) J Clin Microbiol "Detection of
Genital Herpes Simplex Infections by a Tissue Culture
fluorescent - Antibody Technique with Biotin-Avidin"
(17)1: 149-154; papillomavirus, see Mounts et al (1982)

10 Proc Natl Acad Sci USA "Viral etiology of juvenile and
adult - onset of squamous popilloma of the larynx"
(79)17:5425-5429; and cytomegalovirus antigens, see
Oefinger et al (1983) American Society of Microbiology
"Characterization of the humoral response of BALB/C

15 mice immunized with human cytomegatovirus," 83rd Annual
Meeting; March 6-11, 1983 Orleans, LA p. 73.

A patented process is described by Bevey et al. in
U.S. Patent No. 4,228,237, the process described involves
a process for detection and determination of a liquid

20 which employs an insoluble phase enzyme immunoassay
wherein an enzyme labeled avidin and a biotin labeled
reagent detect the liquid.

In addition, Parith, U.S. Patent No. 4,298,685
discloses a process for the detection and/or

25 determination of a biological substance in a test
sample, which comprises admixing the test sample, a
predetermined quantity of a soluble enzyme-label for of
the biological substance, and a predetermined quantity
of a soluble biotin tagged antibody raised against the

30 biological substance. The reactants are allowed to
come to equilibrium and then an insolubilized avidin is
added. The resulting solid phase is separated from the
liquid phase and the enzyme activity of either of these
phases is then determined.

35 Any of these enhancement systems are useable in
the present invention.

As used herein, the term "non-A, non-B hepatitis"
refers to a biologically active material which is

distinct from hepatitis A and hepatitis B virus. The
non-A, non-B hepatitis agents of this invention do
not react immunologically with tests for either of the
known viruses.

5   The present inventors have identified a distinct
non-A, non-B virus material. This material has not
been able to be grown in tissue culture and is not
present in stool as previously reported by Maupas.
        The term "kit" as employed herein means a
10   collection of all or some of the chemicals necessary
to do an immunoassay. A diagnostic kit has been
developed which contains a tissue specimen containing
non-A, non-B hepatitis antigen. Tissue source may be
selected from liver tissue containing active virus
15   activity.
        Tissue preservation may be performed by cutting
a source of tissue containing the non-A, non-B hepa-
titis antigen into convenient sizes. Tissue preser-
vation may be performed by standard fixation and
20   embedding techniques. Such procedures may employ
formalin and/or aldehydes as the fixing agent with
formaldehyde and glutaraldehyde being preferred alde-
hydes and paraffin as an embedding agent. Other standard
materials may also be employed within the scope of the
25   invention.
        Alternatively the tissue may be preserved by
standard cryopreservation techniques wherein the tissue
is unfixed and frozen. Prior to preservation the
tissue may be conveniently cut into cubes or wedges
30   having preferred sizes of 1 cubic centimeter or less.
        Once inactivated the tissue is embedded and stored
for future use. The tissues may then be cut into 2 to
6 micron thick sections and stored for future use.
        The tissue sections are now ready for use. The
35   tissue sections may then be analyzed for the presence
or absence of non-A, non-B hepatitis antigen. One
procedure for performing this analysis involves

contacting the tissue specimen with a non-A, non-B antibody, contacting the resultant tissue specimen with an identification system, and determining the presence of non-A, non-B hepatitis antigen present in the tissue

5   sample. This technique is a simple and convenient method for identifying the presence of non-A, non-B hepatitis antigen in infected liver.

To limit non-specific reaction of the biological specimen antibodies to binding sites in the tissue

10 specimen other than non-A, non-B hepatitis antigen, the tissue may be pretreated with a blocking agent. Exemplary blocking agents may be selected from a wide range of material including normal IgG, normal IgM, bovine serum albumin and normal animal serum. The

15 blocking agent may be used in amounts of about 1% to about 25% and preferably 5% to 12% by weight of a buffered solution. Blocking may be conveniently performed by treating the tissue specimen with the blocking agent at room temperature for up to 1 hour

20 and then removing excess blocking agent with buffered solution. The buffered solutions used herein are not critical with preferred buffers having neutral pH and low ionic strength such as phosphate buffered saline pH 7.0-7.5 (0.01 molar phosphate; 0.15 molar saline).

25 The tissue specimen is then contacted with the biological specimen suspected of containing non-A, non-B hepatitis antibody. The antibody may be derived from normally induced human antibody, chimpanzee antibody and other non human primate antibody. In

30 addition, antibody may be derived from animal immunization resulting in polyclonal and/or monoclonal non-A, non-B hepatitis antibody. Contacting may be performed at temperatures up to 45° C for times up to 24 hours and preferably 20 to 37°C for 2 to 24 hours.

35 The identification system used in the present invention must be sufficiently sensitive to detect the

NANB tissue antigens. It has been found that use of an avidin-biotinylated enzyme complex is at least 40-fold more sensitive than two conventional immunoperoxidase techniques according to dilutional sensitivity

5   studies performed with HBV tissue antigens.

Utilization of the avidin/biotin amplified immunoperoxidase staining procedure, in conjunction with a primary convalescent antiserum obtained from a NANB-hepatitis donor, resulted in the observation of NANB

10 virus associated antigen in the hepatocytes of an infected chimpanzee liver. The same human antiserum was not reactive with a number of uninfected control cells, nor with cells infected with hepatitis A virus, hepatitis B virus or cytomegalovirus. Preincubation of

15 this convalescent NANB serum or IgG derived thereof, with an antigenemic serum obtained from a NANB infected chimpanzee, abolished the antibody reactivity. This is also true in alternate systems which employ animal antibody with animal antigen such as human antibody and

20 human antigen, chimpanzee antibody and chimpanzee or human antigen.

According to the invention, the resultant tissue specimen can then be contacted with the biotin labeled antibody and then contacted with an avidin-biotin

25 system with readable label. The procedure for performing is reaction falls within the scope defined by the prior art and is not critical other than order in which the tagging is used.

Exemplary procedures may employ any non-competitive

30 binding process such as the "Sandwich" technique. In this method the components of the binding reaction may comprise the insoluble tissue specimen containing NANB antigen for the NANB antibody and the following reagents:

35   (i) a measured amount of liquid medium suspected of containing NANB antibody;

(ii)   biotin labeled specific binding
       substance for anti NANB antibody;
(iii) avidin; and
(iv)   biotinylated-labeled enzyme.

5   When the insoluble phase is incubated with indivi-
dual reagents in particular sequence, the binding process
can be affected in either two or three incubation periods.
In a three step incubation process, the insoluble
phase is first reacted with the liquid reagent i.e., a
10   measured amount of liquid medium suspected of containing
the NANB antibody. Unreacted reagent is then removed
and biotin labeled specific binding substance for NANB
antibody is added. Following the reaction of the
biotin labeled specific binding substance, unreacted
15   biotin labeled reagent is removed and enzyme labeled
avidin is added. Following the reaction of enzyme
labeled avidin, unreacted reagent is separated from the
insoluble phase and the enzyme activity of either the
insoluble phase or the separated unreacted enzyme
20   labeled avidin is determined by a suitable detection
reaction. The enzymatic activity of the insoluble
phase is directly related to the amount of ligand in
the liquid medium whereas the enzymatic activity of
separated unreacted enzyme labeled avidin is inversely
25   related in the amount of ligand.
       When biotin labeled specific binding substance is
prereacted with enzyme labeled avidin the binding pro-
cess can be affected in two steps by substituting the
biotin labeled reagent bound to enzyme labeled avidin
30   for the individual biotin labeled and enzyme labeled
avidin reagents.
       Biotin-tagged antibody is conveniently prepared by
reaction of a biotin derivative, for instance a biotin
ester derivative such as the N-hydroxysuccinimide ester
35   of biotin, with the antibody. The biotin ester deriva-
tive is dissolved in a polar, aprotic solvent, for

example dimethylformamide, and is then added in a 20 to 300 molar excess to the antibody in 0.01 M to 1.0 M preferably 0.05 M to 0.5 M, phosphate buffer. The phosphate buffer may have a pH 6.5 to pH 8.5.

5   After admixture of the reactants, the reaction is allowed to proceed at a temperature of from 2° to 10° C for a time sufficient for its completion. Normally this takes about 10 hours. After completion of the reaction, the biotin-tagged antibody may be separated

10   from the reaction mixture by standard methods well known in the art, for example by gel permeation chromatography on, for instance, a crosslinked dextran.

The enzyme labeled avidin reagent or biotin reagent of the instant invention is prepared by

15   conventional procedures, such as by covalent coupling of the selected enzyme to avidin or biotin. This covalent linking can be achieved by direct coupling of existing linking groups or by the addition of external bridging molecules. When using horseradish peroxidase

20   blocking of amino groups and hydroxy groups with fluorodmitrobenzene prior to oxidation prevents self-coupling and allows the enzyme to condense with available amino groups present in avidin. Moreover, alkaline phosphatase can be coupled to avidin or biotin

25   by the condensation of free amino groups in the foregoing molecules with aldehyde groups provided by an external bridging molecule such as glutaraldehyde and other polyfunctional bridging compounds.

Although a wide variety of enzymes can be used to

30   prepare the enzyme labeled avidin or biotin reagent, exemplary enzymes include oxidoreductases and hydrolases according to the International Union of Biochemists (I.U.B.) Particularly preferred oxidoreductases include, glucose oxidase and

35   horseradish peroxidase. Particularly preferred hydrolases include, for example, alkaline phosphatase and B-galactosidase.

The determination of enzyme activity in the instant

invention may, for example, be accomplished with a visual detection or microscopic detection of the reaction system suitable for the particular enzyme employed.

5   A suitable method for assaying for non-A, non-B hepatitis antibody present in a biological sample, would involve contacting a tissue specimen suspected of containing non-A, non-B antigen with the biological specimen to permit reaction of the specimen non-A, non-B hepatitis antibodies with the non-A, non-B

10   antigen. If specific antibodies were present binding would occur at the NANB antigen site. The reaction is optimized by incubating the reactants for up to 24 hours at temperatures up to 45° C. Once the reaction is complete the reactive tissue specimen is separated

15   from the non-reactive phase and the resultant tissue specimen is contacted with a biotin-avidin enhancement system as described above.

     The presence or absence of non-A, non-B hepatitis antibody in the sample may then be made by visual

20   observation in a microscope. A positive result indicates the presence of non-A, non-B hepatitis antibody in the sample.

     A suitable method for assaying for non-A, non-B hepatitis antigen present in a biological sample, would

25   involve reacting suspected NANB antigen present in the sample with a known non-A, non-B hepatitis antibody for up to 10 days at temperatures up to 45°C. This reaction may also be performed in two steps. The first step preferably having a higher temperature and shorter

30   reaction time than the second step. A preferred first step could be performed at a temperature from 20 to 45°C for 1 to 5 hours. This first step aids in the formation of the antibody-antigen complex by driving the reaction to the right, that is to complex

35   formation. The second step is then performed to complete the reaction and form a precipitable immune

complex. The second reaction step is performed at a lower temperature than the first step for a longer period of time. Useable temperatures may be from 2°C to 15°C for times up to 15 days. Once the reaction is

5   complete the reactive phase is separated from non-reactive phase by precipitating immune-complex antibody - antigen and recovering the fluid supernatant. The immune-complex may be precipitated by standard techniques such as centrifugation or reaction

10 with precipitating agents, for example with second antibody, polyethyleneglycol and so forth.

A tissue specimen containing non-A, non-B antigen is then contacted with the fluid supernatant to permit reaction of any non-precipitated non-A, non-B hepatitis

15 antibodies with the non-A, non-B insoluble tissue hepatitis antigen. The reactants are preferably incubated for up to 24 hours at temperatures up to 45° C.

The resultant tissue specimen is then contacted with a biotin - avidin enhancement system described

20 above. The presence or absence of non-A, non-B hepatitis antigen in the sample may then be made by visual observation in a microscope. A negative result indicates the presence of non-A, non-B hepatitis antigen in the sample.

25 This invention also comtemplates a kit for assaying a specimen for a non-A, non-B hepatitis agent which may contain all or some of the materials including a positive tissue sample containing non-A, non-B hepatitis antigen. Preferable the kit contains a tissue specimen

30 which may be fixed and embedded with the non-A, non-B hepatitis agent.

The kit may additionally contain one or more of the following: a quantity of non-A, non-B antibody; a quantity of biotinylated antibody against said non-A,

35 non-B antibody which is derived from a host distinct from the host used to produce the non-A, non-B antibody;

a quantity of avidin and/or biotinylated linked enzyme, and a substrate for the determination of the enzyme activity of said enzyme.

The present invention is further illustrated by
5 the following examples. All parts and percentages in the examples as well as in the specification and claims are by weight unless otherwise specified.

EXAMPLES

Chimpanzee Materials

Liver biopsy material was obtained from chimpan-
zees infected with human plasma containing infectious
5   NANB hepatitis virus. Liver wedge biopsies were
obtained from each of the animals at a time during
which liver enzyme profiles were elevated and patho-
logical indicators of viral hepatitis were noted. Sera
were collected from each of these animals prior to
10   experimental infection with NANB virus, as well as at
weekly intervals after infection for documentation of
liver enzyme changes, and establishment of the acute
disease phase. In addition, sera were also procured
from a number of 2-3 year old juvenile chimpanzees, for
15   use as negative controls.

Human Materials

Liver tissue was obtained at autopsy from a
chronic carrier of HBV. The histopathology of the
specimen was consistent with a diagnosis of chronic
20   hepatitis with cirrhosis. Immunocytochemical tech-
niques had previously documented the presence of HBsAg
and HBcAg in the hepatocytes of this tissue. Normal
control liver tissue was procured which had no
serological markers of HBV infection and the tissue
25   contained no detectable HBsAg or HBcAg by immuno-
cytochemical analysis.

Sera were obtained from several human donors for
these immunocytochemical staining studies. A serum
was obtained from a patient who had recovered from NANB
30   implicated viral hepatitis approximately 7 months after
his liver enzyme profile had returned to normal and
during which time no clinical symptoms indicative of
disease were noted. Sera were absorbed with acetone
extracts of liver powder in order to eliminate
35   nonspecific immunocytochemical reactions. Human or
canine liver powders are rehydrated in phosphate

buffered saline, and incubated with an equal volume of
test serum for one hour. After a second adsorption,
the serum was clarified by centrifugation at 3000 rpm
and filtered through a 0.22 micron filter.

5   Two human antisera were utilized in a study
designed to compare the relative sensitivities of
three different staining techniques for the detection
of HBcAg and HBsAg in a formalin-fixed and paraffin-
embedded liver tissue described below. The first
10   human serum was obtained from an HBV chronic carrier
with antibody to HBcAg (anti-HBc) as detected by solid
phase RIA, but with no detectable antibody to HBsAg
(anti-HBs). The second serum was taken from a hemo-
pheliac who had circulating antibody to both HBsAg and
15   HBcAg. Additional normal control sera were obtained
from donors who had no serological or clinical evidence
of infection with HAV, HBV, or NANB virus.

Tissue Preservation

        Fresh human and chimpanzee liver tissues were cut
20   into 1 cm cubes, washed in cold phosphate buffered
saline (PBS), pH 7.2 and fixed 18-24 hours in 10%
buffered formalin at room temperature. The tissues
were dehydrated through a graded ethanol series,
through toluene, and finally infiltrated and embedded
25   in paraffin, as described previously. The paraffin-
embedded tissues were then cut in 4-5 micron thick
sections on a microtome.

Immunocytochemistry

        Three immunocytochemical procedures were evaluated
30   as described below.

1.   Indirect Immunoperoxidase (IP)

        An indirect immunoperoxidase procedure was per-
formed with the immunoconjugate of goat anti-human.
IgG-horseradish peroxidase (HRPO) used as a second
35   antibody which was diluted 1:10 in a solution of 10%
normal goat serum in PBS (10% NgtS-PBS).

2. __Staphylococcal Protein-A Peroxidase-anti-per__0145489 2
__(Staph A-PAP)__

The staph A-PAP procedure employed was originally
described by Notani et al (1979) __J Histochem Cytochem__

5 "Versatility of Staphylococcus Aureus Protein A in
Immunocytochemistry." 27:1138-1444 which procedure used
a rabbit peroxidaseantiperoxidase.

3. __Avidin-Biotin Immunoperoxidase (ABC-IP)__

The avidin-biotin-immunocytochemical procedure

10 was performed according to general method described
by Hsu et al (1981) __J. Histochem Cytochem__ "Use of
avidin-biotin-peroxidase complex (ABC) in immunoperoxi-
dase techniques." (29)4:577-580. A secondary biotiny-
lated goat anti-human IgG and an avidin-biotinylated

15 horseradish peroxidase complex (ABC) was used.

The procedure involved taking tissue sections
which were pretreated with 10% NgtS-PBS, drained, and
incubated with the individual diluted chimpanzee or
human sera. All primary sera were diluted in 10%

20 NgtS-PBS, and incubations were maintained at room
temperature throughout the procedure. After washing
with cold PBS, the tissue sections were next incubated
with an optimal dilution (1:400 - 1:800 in 10% NgtS-
PBS) of biotinylated goat anti-human IgG. After a 30

25 minute incubation, the sections were again washed, and
incubated with the ABC complex. Excess reagents were
removed from the tissue sections by 3 washes with 0.05M
tris HCl pH 7.6 (Tris buffer). The liver sections were
then reacted in the dark with a substrate solution

30 containing 0.5 mg/ml diaminobenzidine-(HCl)$_4$,
containing 0.01% H_2O_2 in Tris buffer. Selected
specimens were also counterstained with hemotoxylin.
The sections were further dehydrated in ethanol,
transferred into toluene and mounted.

35 __Titration Studies__

Reactive antibody from human donor serum (heat
inactivated at 56° C for 60 minutes) was titrated by
serial two-fold dilution from 1:2 to 1:128 in normal

human serum, and screened against NANB-infected
chimpanzee liver by the ABC-IP procedure. All serum
dilutions were screened for the presence of IgG
antibody as well as IgM antibody by reacting with
5 biotinylated goat anti-human IgM.

To confirm the antibody activity observed in the
donor serum, purified IgG from donor serum was screened
and titrated as above. Purification of the globulins
was accomplished by addition of $(NH_4)_2SO_4$, and the
10 IgG fraction was further purified by DEAE cellulose ion
exchange chromatography.

Blocking Studies

Reactive antibody samples were screened again by
the ABC-IP procedure following pre-incubation with
15 acute phase serum from chimpanzees or from human
patients infected with NANB hepatitis in order to
immunologically block the antibody activity. Immu-
nological blocking was evidenced by a considerable
diminution or complete elimination of the immunoperoxi-
20 dase staining. Sample aliquots of donor serum (100 ul)
were incubated with an equal volume of presumably
antigenemic serum (chimp or human) for 2 hours at 37C,
followed by an overnight incubation at 4°C. At the end
of the second incubation all samples were centrifuged
25 at 5,000 rpm for 30 minutes at 4°C and the supernatant
serum protein titrated for residual antibody activity.
Control samples for the blocking studies included
normal human serum, young (2-3 yr.) juvenile chimpanzee
serum, chimpanzee pre-inoculation sera, and heat
30 inactivated (56°C, 1 hour) acute phase chimpanzee serum.

Relative Sensitivities of Immunocytochemical Methods

HBV reagents, i.e., human sera containing antibody
to HBV antigens of a HBV antigen positive human liver,
were utilized to evaluate the relative sensitivity
35 levels of the three immunocytochemical staining
procedures. The three techniques used were the IP, the

Staph A-PAP and the ABC-IP. The two human sera
materials described containing either antibody to HBcAg
only or antibody to both HBcAg and HBsAg, were tested
undiluted and at 2-fold dilutions with tissue sections
5 obtained from a chronic HBV infected liver, and with
tissue from a normal, uninfected human liver. No
staining of the infected liver tissue section was noted
using either sera as primary reagents in the IP
procedure. However, a weak reaction was noted with
10 both sera in the nuclei of isolated hepatocytes with
the staph A-PAP method at sera dilutions of 1:2 and 1:4
in the absence of cytoplasmic staining. The same
reagents were also used to test the sensitivity of the
ABC-IP staining procedure. At a serum dilution of 1:80
15 both sera stained the nuclei of the majority of cells
in isolated hepatic lobules. The human serum obtained
from a hemophiliac patient which contained both anti-
HBc and anti-HBs activity, also stained the cytoplasm
of isolated hepatocytes as well as cell nuclei at a
20 serum dilution of 1:40. Neither serum stained liver
tissue procured from an individual negative for HBV
serologic markers using the ABC-IP method. In
addition, serum obtained from a control individual was
negative for anti-HBs and anti-HBc failed to react with
25 the infected liver tissue.
Screening of NANB Infected Liver With Anti-NANB Serum
Liver tissue sections were procured from
chimpanzees with pathological evidence for NANB
hepatitis infection. These tissues were screened
30 against heat treated donor serum by the ABC-IP immuno-
cytochemical procedure for anti-NANB/NANB antigen
reaction. The ABC-IP procedure yielded consistent,
positive evidence for cytoplasmic antigen reactivity.
Intense cytoplasmic staining was visible in 7-10% of
35 the hepatocytes. Control liver specimens obtained from
normal (i.e., non-infected) chimpanzee and reacted with

heat treated donor serum were consistently negative for cytoplasmic staining. Similarly, normal human liver, HBV-infected human liver, and HAV-infected chimpanzee liver were all negative for cytoplasmic staining when

5 reacted with donor serum. As a final control, normal human serum was unreactive with NANB-infected chimpanzee liver. The cytoplasmic localization of the specific stain reaction was clearly demonstrated. Adsorption of donor serum with canine or normal human

10 liver powder did not diminish the immunoreaction with the NANBinfected chimpanzee liver.

Titration of Antibody Activity to NANB Antigen

An aliquot of several donor serum, procured approximately seven months into convalescence (i.e., after all

15 chemical and clinical symptoms returned to normal), was titered by serial two-fold dilutions from 1:2 through 1:128 and screened against NANBinfected chimpanzee liver. Positive staining was observed in the cytoplasm and scored as either intense (++), moderate (+), or

20 negative (-). The serum titer of anti-NANB was 1:32, with moderate staining observed (Table I). In a second group of titration experiments, IgG, derived from donor serum, was diluted as described above and screened against NANB-infected chimpanzee liver. An endpoint

25 titer of 1:16 was observed when scored as above (Table I).

TABLE I

Titration of Antibody Activity To NANB Antigen

Antibody Reagent	Un-dilute	Dilution						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
Donor Serum Antibody #1	++	++	++	++	++	+	−	−
Donor Purified IgG #1	++	++	++	+	+	−	−	−
Donor Serum Antibody #2	++	++	++	+	−	−	−	−
Donor Serum Antibody #3	++	++	++	++	++	++	·+	−
Donor Serum Antibody #4	++	++	++	+	−	−	−	−

Blocking Studies

The specificity of the observed cytoplasmic
stain reaction was investigated by immunological
blocking of donor serum antibody activity with
5 chimpanzee or human implicated NANB acute and chronic
sera. Donor serum stain activity was not blocked by
pre-incubation with either preinoculation chimpanzee
serum, or normal human serum, normal chimpanzee serum,
or young juvenile chimpanzee serum. However, preincu-
10 bation of donor serum with either of two acute phase
NANB chimpanzee sera or with chronic phase NANB human
serum, effectively blocked antibody activity. In
addition, antibody activity in purified donor IgG was
blocked with these antigenemic sera. Results of all
15 blocking studies are summarized in Table II.

TABLE II

Antibody pre-incubated with serum from:	Residual actibody reactivity in:	
	Serum	IgG
Normal human	++	++
Normal chimp	++	++
Juvenile chimp	++	++
Pre-inoculation chimp[b]	++	++
Acute chimp A[b]	-	-
Acute chimp B[b]	-	-
Chronic human[b]	-	-

Cytoplasmic Staining was scored as intense (++),
moderate (+), or negative (-). The b. notation
30 indicates serum obtained from chimpanzees before and
after inoculations with NANB or from a NANB infected
human.

A summary of the above staining patterns was
demonstrated by a number of control experiments. The
35 results indicated that donor serum did not react with
liver tissue from normal uninfected chimpanzee or

human. In addition, no reaction was noted when the
same serum was used to stain sections of HAV- or
HBV-infected liver tissue. Normal chimpanzee or human
serum did not stain the NANB-infected tissue by the

5 ABC-IP method. The most convincing evidence for
specificity was obtained by preincubation of donor
serum with either of two different sera from two
chimpanzees derived during the acute phase of their
NANB infection. Preincubation with either of these two

10 presumably antigenemic sera completely blocked the
staining reaction, whereas, preincubation with normal
chimpanzee or human serum had no observable effect on
the intensity of the staining. These results indicated
the specificity of the ABC-IP reaction for NANB

15 associated antigens.

The invention being thus described, it will be
obvious that the same may be varied in many ways. Such
variations are not to be regarded as a departure from
the spirit of scope of the invention and all such

20 modifications are intended to be included within the
scope of the claims.

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CLAIMS:

1. A process for assaying a tissue specimen for non-A, non-B hepatitis antigen, which process comprises:

    (a) providing a tissue specimen to be assayed;

    (b) contacting the tissue specimen with a non-A, non-B hepatitis antibody;

    (c) contacting the resultant tissue specimen with a biotin-avidin system; and

    (d) determining the presence of non-A, non-B hepatitis antigen present in the tissue sample.

2. A process according to Claim 1, wherein the tissue specimen is a piece of liver tissue.

3. A process according to Claim 2, wherein the tissue specimen is fixed and embedded.

4. A process according to Claim 2, whrein the tissue specimen is unfixed, frozen tissue.

5. A process according to any preceding claim, wherein the tissue specimen is pretreated or otherwise contacted with a blocking agent to block nonspecific binding sites, the blocking agent preferably being chosen from: normal IgG, normal IgM, bovine serum albumin, and normal animal serum.

6. A process according to any preceding claim, wherein the non-A, non-B hepatitis antibody is IgG or IgM.

7. A process according to any preceding claim, wherein the tissue specimen is contacted with the non-A, non-B hepatitis antibody at a temperature of up to 45°C, preferably from 20°C to 37°C, for a time of up to 24 hours, preferably from 2 to 24 hours.

8. A process according to any preceding claim, wherein the non-A, non-B hepatitis antibody

of step (b) is biotinylated prior to its contacting
with the tissue specimen.

9.   A process according to any preceding
claim, wherein the resultant tissue specimen

5   of step (c) is contacted with a biotin labelled
antibody against the non-A, non-B hepatitis antibody
which biotin labelled antibody is derived from
a host distinct from the host sued to produce
the non-A, non-B hepatitis antibody.

10   10.   A process according to any one of Claims
1 to 7, wherein the resultant tissue specimen
of step (c) is first contacted with a biotin labelled
antibody and then contacted with readable labelled
avidin-biotin system.

15   11.   A process for assaying for non-A, non-B
hepatitis antibody present in a biological sample,
which process comprises:

        (a)   contacting a tissue specimen containing
non-A, non-B hepatitis antigen with the biological

20   specimen to permit reaction of the specimen non-A,
non-B hepatitis antibodies with the non-A, non-B
hepatitis antigen;

        (b)   incubating the reactants for a time
of up to 24 hours at a temperature of up to 45°C;

25   (c)   separating the tissue specimen from
the non-reactive phase;

        (d)   contacting the resultant tissue specimen
with a biotin-avidin system; and

        (e)   determining the presence of non-A,

30   non-B hepatitis antibody in the sample.

        12.   A process according to Claim 11, wherein
a positive result indicates the presence of non-A,
non-B hepatitis antibody in the sample.

        13.   A process for assaying for non-A, non-B

35   hepatitis anitgen present in a biological sample,
which process comprises:

        (a)   reacting antigen present in the sample

with non-A, non-B hepatitis antibody for a time
of up to 10 days at a temperature of up to 45°C;

(b) separating reactive phase from non-reactive
phase by precipitating immune-complexed antibody
- antigen and recovering the liquid supernatant;

(c) contacting a tissue specimen containing
non-A, non-B hepatitis antigen with the liquid
supernatant to permit reaction of any non-precipitated
non-A, non-B hepatitis antibodies with the non-A,
non-B hepatitis antigen present in the tissue
specimen;

(d) incubating the reactants for a period
of up to 24 hours at a temperature of up to 45°C;

(e) contacting the resultant tissue specimen
with a biotin-avidin system; and

(f) determining the presence of non-A,
non-B hepatitis antigen in the sample.

14. A process according to Claim 13, wherein
a negative result indicates the presence of non-A,
non-B hepatitis antigen in the sample.

15. A process according to Claim 13 or
14, wherein the reaction of anitgen with antibody
in step (a) is performed in two steps, the first
step being at a temperature of from 20 to 45°C
for from 1 to 5 hours and a second step at a
temperature lower than the temperature of the
first step and for a time longer than that of the
first step.

16. A tissue specimen which is fixed and
embedded and which contains non-A, non-B hepatitis
antigen.

17. A diagnostic kit which comprises:

(a) a tissue specimen which is fixed and
embedded and which specimen contains non-A, non-B
hepatitis antigen;

(b) a quantity of non-A; non-B hepatitis
antibody;

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(c)   a quantitiy of a biotin-avidin system; and

(d)   a substrate for the determination of
the enzyme activity of the enzyme.

18.   A diagnostic kit according to Claim

5   17, wherein the biotin-avidin system comprises
a biotin labelled antibody and a readable labelled
avidin-biotin system.

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